

## REMARKS/ARGUMENTS

Claims 1-8 remain herein. Claims 9-42 and 58-131 have been withdrawn from consideration by the U.S. PTO. Claims 43-57 are canceled hereby. New claims 132-146 are added hereby. Thus, claims 1-42 and 58-146 are pending herein.

Claims 1-8 were rejected under 35 U.S.C. §102(e) over U.S. Patent No. 5,795,748 (Cottingham '748). In addition, claims 1-8 were rejected under 35 U.S.C. §102(e) over U.S. Patent No. 5,948,673 (Cottingham '673).

Neither Cottingham '748 nor Cottingham '673 discloses or suggests a method which comprises loading a sample portion into a sample chamber, in which if the sample portion contains a *single molecule* of a target nucleic acid, the sample portion would attain a detectable concentration of the target nucleic acid within a portion of the sample chamber after a *single round* of amplification.

Cottingham '748 discloses that Figure 5B therein shows a sample well assembly 22 fully assembled with a liquid biological sample 60 present in the capillary chamber 54, and that the liquid biological sample 60 substantially fills the capillary chamber 54, which has a volume of about 20  $\mu$ L (Cottingham '748, col. 6, lines 50-54). Cottingham '748 discloses that the liquid biological sample is drawn into the chamber 54 by capillary forces and is spread into a thin film or disk having a height (thickness) of about 0.020 inch and a diameter of about 0.250 inch (Cottingham '748, col. 6, lines 56-60).

Cottingham '673 discloses a device which includes a multiplicity of discrete sample cells, and that the volume of the liquid biological sample will typically be about 20  $\mu$ L (Cottingham '673, col. 6, lines 55-57).

In a 20  $\mu$ L volume, amplified product from a *single target molecule* would not be detectable.

Attached is a copy of a paper entitled "Nanoliter scale PCR with TaqMan detection",  
Nucleic Acids Research (Kalinina et. al., 1997), which provides strong evidence that the  
amplified product of a single molecule could not be detected in 20 microliter volumes.

Accordingly, it is respectfully requested that the U.S. PTO reconsider and withdraw  
these rejections.

In view of the above, claims 1-8 and 132-146 are in condition for allowance.

If the Examiner believes that contact with Applicant's attorney would be advantageous  
toward the disposition of this case, the Examiner is herein requested to call Applicant's  
attorney at the phone number noted below.

The Commissioner is hereby authorized to charge any additional fees associated with  
this communication or credit any overpayment to Deposit Account No. 50-1446.

Respectfully submitted,



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# Nanoliter scale PCR with TaqMan detection

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## ABSTRACT

We monitored PCR in volumes of the order of 10 nl in glass microcapillaries using a fluorescence energy transfer assay in which fluorescence increases if product is made due to template-dependent nucleolytic degradation of an internally quenched probe (TaqMan assay). This assay detected single starting template molecules in dilutions of genomic DNA. The results suggest that it may be feasible to determine the number of template molecules in a sample by counting the number of positive PCRs in a set of replicate reactions using terminally diluted sample. Since the assay system is closed and potentially automatable, it has promise for clinical applications.

## INTRODUCTION

While PCR has had a major impact on molecular biology research, its application to clinical diagnostics has been slow, due in part to: (i) labor intensiveness of methods for detecting PCR product; (ii) susceptibility of PCR to 'carry-over contamination', i.e. false positives due to contamination of a sample with molecules amplified in a previous PCR; (iii) difficulty using PCR to quantitate the number of target nucleic acid molecules in a sample.

Recently, significant progress has been made on these problems with the introduction of the 'TaqMan' fluorescence energy transfer assay (1,2). This assay uses a nucleic acid probe complementary to an internal segment of the target DNA. The probe is labeled with two fluorescent moieties with the property that the emission spectrum of one overlaps the excitation spectrum of the other; as a result the emission of the first fluorophore is largely quenched by the second. The probe is present during PCR and if PCR product is made, the probe becomes susceptible to degradation via a 5'-nuclease activity of Taq polymerase that is specific for DNA hybridized to template ('TaqMan' activity). Nucleolytic degradation of the probe allows the two fluorophores to separate in solution, which reduces quenching and increases the intensity of emitted light. Because this assay involves fluorescence measurements that can be performed without opening the PCR tube, the risk of carry-over contamination is greatly reduced. The TaqMan assay is not labor intensive and is easily automated.

The TaqMan assay has also provided a method for quantitation of target nucleic acids. Early methods of quantitative PCR relied on setting up PCRs with known numbers of target nucleic acid molecules and comparing the amount of PCR product generated

from these control reactions to that generated from an unknown sample (reviewed in 3). Later versions of this method used an 'internal control', i.e. a target nucleic acid added to the PCR that should amplify at the same rate as the unknown but which could be distinguished from it by virtue of a small sequence difference (e.g. a small insertion or deletion or a change that led to the gain or loss of a restriction site or reactivity with a special hybridization probe) (4,5). These methods have the disadvantage that slight differences in amplification efficiency between the control and experimental nucleic acids can lead to large differences in the amounts of their products after the million-fold amplification characteristic of PCR and it is difficult to determine relative amplification rates accurately. Newer quantitative PCR methods use the number of cycles needed to reach a threshold amount of PCR product as a measure of the initial concentration of target nucleic acid, with ethidium bromide (6) or TaqMan assays (7,8) used to follow the amount of PCR product accumulated in real time. However, these assays also require assumptions about relative amplification efficiency in different samples during the exponential phase of PCR.

An alternative method of quantitation is to determine the smallest amount of sample that yields PCR product, relying on the fact that PCR can detect a single template molecule. However, to achieve single molecule sensitivity, two or more sequential PCRs usually have to be performed, often using nested sets of primers, and this accentuates problems with carry-over contamination.

We reasoned that the sensitivity of the TaqMan assay could be improved to enable detection of single starting molecules if reaction volumes were reduced. The TaqMan assay requires near saturating amounts of PCR product to detect enhanced fluorescence (see below). PCRs normally saturate at  $\sim 10^{11}$  product molecules/ $\mu$ l, due in part to rapid reannealing of product strands. To reach this concentration of product after 30 cycles in a 10  $\mu$ l PCR requires at least  $10^3$  starting template molecules ( $10^3 \times 2^{30}/10 \mu\text{l} = 10^{11}/\mu\text{l}$ ). Somewhat less than this number of starting molecules can be detected by increasing the number of cycles and in special circumstances even single starting molecules may be detectable (9), but this strategy usually fails before getting to the limit of detecting single starting molecules due to the appearance of artifactual amplicons derived from the primers (so called 'primer dimers'). However, if the PCR volume were reduced to 10 nl, then a single target molecule might suffice to generate a saturating concentration of PCR product after  $\sim 30$  cycles ( $1 \times 2^{30}/10 \text{ nl} = 10^8/\text{nl} = 10^{11}/\mu\text{l}$ ). Fluorescence changes in the TaqMan assay can be detected in small volumes using a fluorescence microscope. Therefore, we investigated whether volume reductions would

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allow detection of PCR products generated from single target molecules using the TaqMan fluorescence energy transfer system.

## MATERIALS AND METHODS

### Molecular biology reagents

We used a TaqMan kit from Applied Biosystems (Foster City, CA). This kit contains human DNA at 10 ng/ $\mu$ l, forward and reverse primers (5'-TCACCCACACTGTGCCCATCTACGA-3' and 5'-CAGCGGAACCGCTCATTGCCAATGG-3' respectively) that amplify a 295 bp segment of the human  $\beta$  actin gene and a dual fluorophore-labeled probe [5'-(6-carboxyfluorescein)-ATGCCC-(6-carboxytetramethylrhodamine)-CCCCATGCCATC-CTGCGT-3'] that is complementary to bases 31–56 of the PCR product. (For simplicity, we refer to 6-carboxyfluorescein as 'fluorescein' and 6-carboxytetramethylrhodamine as 'rhodamine'.) We used Taq polymerase from Boehringer Mannheim (Indianapolis, IN) and anti-Taq antibody from Clontech (Palo Alto, CA). PCRs contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% gelatin, 500  $\mu$ g/ml-5 mg/ml bovine serum albumin (BSA), 3.5 mM  $MgCl_2$ , 0.2 mM each dATP, dCTP, dGTP and dUTP, 0.3  $\mu$ M forward and reverse primers, 0.2  $\mu$ M dual fluorophore-labeled probe, 0.5 U Taq polymerase/10  $\mu$ l PCR mixture, 0.1  $\mu$ l anti-Taq antibody/10  $\mu$ l PCR mixture and varying amounts of template DNA. The specific activity of Taq polymerase was ~250 000 U/mg, which, at a molecular weight of 100 kDa, translates to ~10<sup>9</sup> molecules/ $\mu$ l. Since  $\beta$  actin is a single copy gene, we estimated one copy of  $\beta$  actin template/3 pg human genomic DNA. In some PCRs the dual fluorophore-labeled probe was replaced with the fluorescent, DNA staining dye SYBR<sup>TM</sup> Green I (product no. S-7567; Molecular Probes, Eugene, OR) used at a 10<sup>-4</sup> dilution from the stock supplied by the manufacturer.

### PCR apparatus

Conventional PCRs were performed in 0.2 ml polypropylene tubes in a model 9600 thermocycler from Perkin Elmer using 92°C for 15 s, 54°C for 15 s, 72°C for 15 s for 40 cycles.

Capillary PCR was performed in quartz glass microcapillaries from Polymicro Technologies (Phoenix, AZ). These capillaries had inner diameters of 20–75  $\mu$ m and outer diameters of 250–375  $\mu$ m. The capillaries come with either a polyimide or teflon external coating to make them flexible. Because the polyimide coating is opaque and fluorescent, it had to be removed before use. This was done by flaming a segment of polyimide capillary with a Bunsen burner for several seconds and then gently wiping off the burned coating; this was repeated as necessary until the capillary was clear. The resulting bare capillaries are quite fragile. We found that the teflon-coated capillaries were easier to work with, since the optically clear and non-fluorescent teflon coating did not need to be removed. Both types of capillaries gave equivalent results in PCR.

Capillaries were filled by touching an open end to a drop of PCR solution which wicked in by capillary action. Capillaries were sealed and supported by gluing each end to a different coverslip, leaving an unsupported segment in the middle to minimize thermal mass (see Fig. 1). The glue (optical adhesive no. 81; Norland Products, New Brunswick, NJ) was cured by exposure to 366 nm UV light with a UV lamp (model UVL-21; UVP Inc., San Gabriel, CA) held ~1 cm from the sample for 30 s. The PCR mixture was shielded from UV light by laying a small

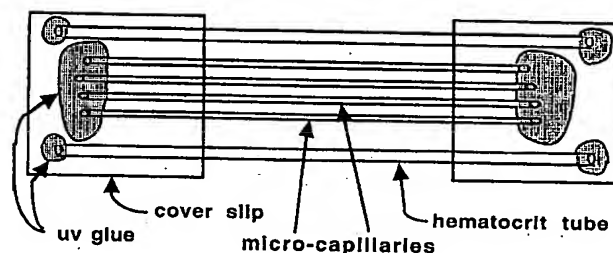


Figure 1. Schematic diagram of microcapillary PCR sample holding device.

piece of opaque paper over the center section of the capillary during UV exposure. For structural support, one or two hematocrit capillary tubes were glued to the coverslips. This sample holding device was then attached with Scotch<sup>®</sup> tape to the sample holder of a Rapidcycler air oven (Idaho Technologies, Idaho Falls, ID) and cycled through 92°C for 5 s, 54°C for 5 s, 72°C for 15 s for 40 cycles; this cycling protocol takes ~30 min in the Rapidcycler.

### Fluorescence detection

Fluorescence of samples in glass capillaries was measured with a Zeiss axiovert 410 laser scanning microscope using a 20X-0.5NA objective, 15 mW external argon laser, ~5% of the power of which was focused to a spot size of 1  $\mu$ m<sup>2</sup>, and bandpass filters of 515–565 nm for fluorescein and SYBR<sup>TM</sup> Green and >590 nm for rhodamine (power loss and spot size estimates provided by the manufacturer). Average pixel intensity was measured in regions of ~20 × 50  $\mu$ m overlying the capillary image. Fluorescence intensity was examined visually along the length of 2.5 cm capillary tubes by manually translating the stage; quantitative measurements were made every 2–5 mm, or more often if variability in the fluorescence signal was observed.

## RESULTS

When PCRs were performed in 20  $\mu$ l volumes in Eppendorf tubes in a model 9600 Thermocycler (Perkin Elmer, Norwalk, CT), the  $\beta$  actin primers amplified an ~300 bp segment from human DNA as expected. PCRs performed in the presence of the TaqMan probe were transferred to capillary tubes and analyzed by fluorescence microscopy. Typical values for average pixel intensity were ~130 relative fluorescence units (RFU) for fluorescein and ~60 RFU for rhodamine, with background emission from empty capillaries ~20 RFU at both wavelengths. In different experiments the fluorescein:rhodamine (F/R) ratio varied from ~1.0 to 2.0 in samples containing PCR product. For negative control PCRs containing no template DNA, no Taq polymerase or no reverse primer, the rhodamine emission was about the same (~60 RFU), while the fluorescein emission was reduced to ~30 RFU, giving a F/R ratio of ~0.5. The absolute values of fluorescein and rhodamine emission varied between experiments and with small changes in machine settings (laser power, attenuation, brightness, contrast), whereas the F/R ratio was fairly constant; therefore the latter was used as a measure of whether the  $\beta$  actin product had been amplified.

We estimated the yield of PCR product in conventional reactions in polypropylene tubes by ethidium bromide staining of product in agarose gels and by adding a known amount of [<sup>32</sup>P]dCTP to a PCR and counting radioactivity in the purified

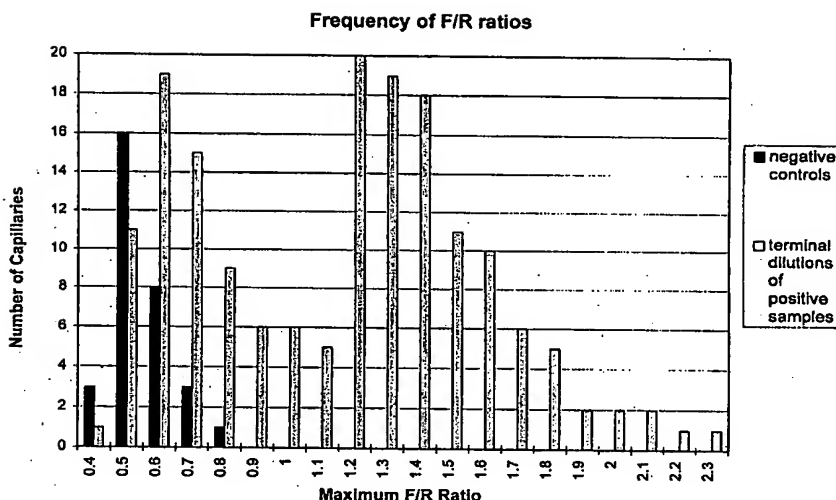


Figure 2. Histogram of maximum F/R values in capillaries containing negative control PCRs (black bars) and PCRs with terminal dilutions of human genomic DNA (gray bars).

PCR product. Both methods gave an estimate of  $\sim 10^{11}$  product molecules/ $\mu$ l PCR. This corresponds to a product concentration of  $\sim 0.16 \mu$ M, which implies that about half of the PCR primers were converted to product.

To assess the extent of degradation of the TaqMan probe following PCR, we examined the effect of mung bean nuclease on the F/R ratio. Treatment of the probe with mung bean nuclease for 10 min at  $37^\circ\text{C}$  raised the F/R ratio from 0.5 to 5. This presumably represents complete degradation, since further incubation did not increase the ratio. An F/R ratio of 1.5, characteristic of positive PCRs, therefore suggests that  $\sim 30\%$  of probe was degraded. This corresponds to a concentration of degraded probe of  $0.06 \mu$ M and implies that about one third of the probe that could have hybridized to PCR product was degraded.

When PCRs were performed in small diameter glass capillaries, the volume of the reaction was too small to detect PCR product by standard gel electrophoresis. While products might have been detected by capillary electrophoresis (10,11), we were interested to see if the TaqMan assay could be used as an alternative. The F/R ratio was  $\sim 0.5$  in negative control reactions (no template, no enzyme or no reverse primer) and was usually  $>1$  in samples where product was expected. In capillaries containing terminal dilutions of genomic DNA template, the ratio sometimes varied with position along the capillary, which we attributed to localized accumulation of degraded probe (see below). In these cases we used the maximum recorded value of the F/R ratio in a capillary as the measure of whether the target sequence had been amplified.

A histogram of the maximum values of the F/R ratio in over 100 capillary reactions of terminally diluted genomic DNA is shown in Figure 2, along with the corresponding values for negative control reactions. The negative controls had a mean ratio of 0.5, with a range of 0.4–0.9. The experimental samples had a bimodal distribution, with one arm of the distribution paralleling that of the negative control samples. This suggests that the experimental samples consisted of positive and negative samples. Since the

nadir of the experimental sample distribution occurred at an F/R ratio of  $\sim 1$ , we chose this value as a 'cut-off' to distinguish positive from negative samples. This 'cut-off' is consistent with the F/R values of 1–2 in PCRs carried out in conventional volumes in Eppendorf tubes.

Using a 'cut-off' of  $F/R \geq 1$ , we estimated the sensitivity of our detection system by mixing exonuclease-digested probe with undegraded probe. An F/R ratio of  $\geq 1$  was obtained when  $\geq 0.02 \mu$ M degraded probe was mixed with  $0.2 \mu$ M undegraded probe. This corresponds to  $\sim 10^8$  molecules of degraded probe in a 10 nl volume. Using the confocal feature of the microscope, we found that the signal decreased rapidly when the depth of field dropped below  $20 \mu\text{m}$ . Thus, an estimate of the lower limit of detection for this system is  $\sim 10^5$  molecules of degraded probe in a volume of  $20 \times 20 \times 20 \mu\text{m} = 10 \text{ pl}$ .

As has been noted by others performing PCR in glass tubes (12), it was essential to include BSA in the PCRs, presumably to block non-specific adsorption of DNA to glass. When BSA was not included, the F/R ratio was  $\sim 0.5$ . We generally used  $500 \mu\text{g/ml}$  final concentration of BSA in the PCRs, although for some batches of BSA we had to increase the concentration to  $5 \text{ mg/ml}$ .

Human DNA was diluted so that PCRs contained 0–14 haploid genome equivalents (0–42 pg/capillary). Reactions were scored as positive if the maximum F/R ratio along the tube was  $\geq 1.0$ . The results for a series of PCRs in capillaries with internal diameters of 20–75  $\mu\text{m}$  are shown in Table 1 and Figure 2.

Capillaries containing  $>1$  haploid genome equivalent generally had F/R ratios  $>1$ . In capillaries containing  $<1$  haploid genome equivalent, the fraction of capillaries with F/R ratios  $\geq 1$  was roughly proportional to the fraction of capillaries expected to contain one or more template molecules. This fraction was calculated from the Poisson distribution as  $1 - e^{-m}$  where  $m$  is the amount of DNA/capillary/3 pg. These results provide strong support for the hypothesis that the method is sensitive to a single starting template molecule.

Table 1. Replicate PCRs in microcapillaries with terminal dilutions of genomic DNA

Capillary diameter ( $\mu$ )	Haploid genome equivalents/capillary ( $m$ )	Probability of $\geq 1$ template/capillary	Fraction of capillaries with max F/R $> 1$	No. of capillaries
20	0	0.00	0.00	2
	0.2	0.18	0.10	10
	0.5	0.39	0.33	15
25	0	0.00	0.00	4
	0.4	0.33	0.28	18
	0.8	0.55	0.50	8
	1.5	0.78	1.00	3
	3	0.95	1.00	3
30	0	0.00	0.00	8
	0.5	0.39	0.52	23
	1	0.63	0.92	13
	1.5	0.78	1.00	10
50	4	0.98	1.00	3
	0	0.00	0.00	13
	0.4	0.33	0.00	3
	0.8	0.55	0.67	27
	1.5	0.78	0.82	28
	3	0.95	1.00	2
	6	1.00	0.89	9
	13	1.00	1.00	7
75	0	0.00	0.00	7
	0.8	0.55	0.50	6
	1.7	0.82	0.67	12
	3.4	0.97	1.00	9
	7	1.00	1.00	5
	14	1.00	1.00	6

Haploid genome equivalents per capillary,  $m$ , was calculated by dividing the amount of DNA/capillary (in pg) by 3 pg/haploid genome equivalent. The probability of  $\geq 1$  template/capillary was calculated from the Poisson distribution as  $1 - e^{-m}$ . About one sixth of the PCRs contained low melt agarose at 0.2–0.8%; as the agarose had no detectable effect (see text), the data from capillaries with and without agarose were pooled.

Similar results were obtained with another preparation of human genomic DNA obtained from Promega: at 8 haploid genome equivalents (24 pg/capillary), four of four capillaries gave maximum F/R ratios  $\geq 1$ ; at 0.7 haploid genome equivalents (2 pg/capillary), three of four capillaries were positive; at 0.1 haploid genome equivalents (0.4 pg/capillary), none of four capillaries were positive.

The inhomogeneity of F/R ratio along the length of capillaries containing  $\sim 1$  template molecule suggested that we might be seeing residual localization of degraded probe as a result of localized accumulation of PCR product. To investigate this possibility, we performed amplifications in 2.5 cm capillaries containing  $\sim 0.5$  haploid genome equivalents/capillary. A plot of F/R ratio along a few representative capillaries is shown in Figure 3. Some capillaries have a single peak, others two peaks, suggesting two areas where PCR product and degraded probe had accumulated. The half-widths of the peaks (measured at half-height) were 3–6 mm. When capillaries were left overnight, the distributions broadened and flattened. Inhomogeneities in F/R ratio were not seen when capillaries were examined before PCR or after PCR in capillaries containing no template DNA or  $\sim 75$  initial template molecules. Representative experiments are shown in Table 2. These results argue that the inhomogeneities were not due to smudges blocking light transmission, thermal variations during PCR or photobleaching. We further checked that the 30 s UV irradiation used to seal the ends of the capillaries did not alter the F/R ratio. Photobleaching of the fluorescein (but not the rhodamine) signal was detectable with repeated laser scanning at

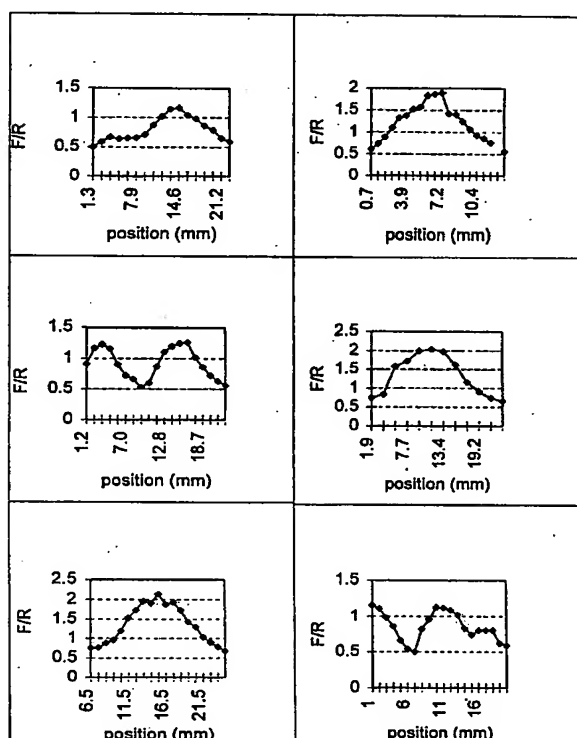
the highest power, with 10 scans reducing the fluorescein signal  $\sim 10\%$ ; however, only one or two scans at this power were performed at any one location when collecting data, so this does not explain the inhomogeneities. To see if convection after PCR might be broadening the peaks, we added 0.2–0.8% low-melt agarose to some PCRs, but no effect of the agarose was noted. A few of the capillaries fortuitously contained air bubbles that divided the sample into two or more segments. In several of these cases, the F/R ratio was  $\geq 1$  on one side of the bubble and 0.5 on the other side, consistent with blocked diffusion of degraded probe.

To confirm the results of the TaqMan assay, we substituted the fluorescent dye SYBR<sup>TM</sup> Green I for the TaqMan probe. Because the fluorescence of SYBR<sup>TM</sup> Green I increases many-fold in the presence of double-stranded DNA, it can be used to detect double-stranded PCR product (13), although it does not distinguish spurious product, such as 'primer dimer', from desired product. The SYBR<sup>TM</sup> Green I fluorescence assay has to be performed at elevated temperature to reduce background fluorescence from non-specific annealing of primers. To do this we placed segments of capillaries, after PCR, in  $\sim 1$  ml mineral oil in a special 35 mm Petri dish, the bottom of which was made of optically clear, conducting glass coated with a thin layer of indium tin oxide (Bioprotech, Butler, PA). By applying 3–4 V across the bottom of the dish, we raised the temperature in the oil to  $\sim 70^\circ\text{C}$ . Because only a portion of the bottom of the Petri dish was flat and accessible in the microscope, we had to cut the capillaries after PCR into  $\sim 1$  cm segments in order to image them.

**Table 2.** High variability in F/R is specific for capillaries with  $\sim 1$  target molecule and decreases with time

Group	Haploid gene equivalents/capillary	Time when fluorescence analysed	No. of capillaries	No. of capillaries with max F/R >1	Average F/R	S.D. of F/R
A	0	1 h after PCR	9	0	0.5	0.05
B	75	1 h after PCR	10	10	1.75	0.10
C	1 (expt 1)	before PCR	5	0	0.51	0.04
D	1 (expt 1)	1 h after PCR	5	4	0.83	0.46
E	1 (expt 1)	24 h after PCR	5	3	0.89	0.23
F	1 (expt 1)	48 h after PCR	5	1	0.82	0.23
G	1 (expt 2)	before PCR	5	0	0.46	0.03
H	1 (expt 2)	1 h after PCR	5	5	1.22	0.43
I	1 (expt 2)	24 h after PCR	5	5	1.14	0.25
J	1 (expt 2)	48 h after PCR	5	5	1.11	0.21

F/R measurements were taken every 2–5 mm along 2.5 cm long capillaries as described in Materials and Methods. Measurements from different capillaries containing aliquots of the same sample were pooled for calculating standard deviation (S.D.).



**Figure 3.** Profile of F/R values along representative capillaries containing  $<1$  haploid genome equivalent of genomic DNA. Positions along the x-axis were estimated by moving the microscope stage manually in approximately equal increments and dividing the capillary length by the number of increments.

Using this device we could detect PCR product derived from single template molecules. For example, the fluorescence intensity was 155–194 RFU in seven capillary segments derived from a PCR containing 30 haploid genome equivalents/cm capillary length, compared with a fluorescence intensity of 40–57 RFU in seven capillary segments containing no template DNA. The variability in fluorescence at 2–3 mm intervals along these capillaries was  $\sim 20\%$ . In contrast, in seven capillary segments

derived from PCRs containing 0.3 haploid genome equivalents/cm capillary, the fluorescence intensity varied from 49 to 136 RFU, with four capillary segments having fluorescence intensity  $<73$  RFU at all tested positions along their lengths, two capillary segments having fluorescence intensity  $>100$  RFU at all positions and one capillary having a fluorescence intensity of 68 RFU at one end increasing to 122 RFU at the other end. These results provide additional evidence that PCR products derived from single molecules can be detected in small volumes.

## DISCUSSION

The results presented here show that the TaqMan assay can detect as little as 1 template molecule when the volume of the reaction is of the order of 10 nl. For 50  $\mu\text{m}$  inner diameter capillaries, the reaction volume is  $\sim 20$  nl/cm capillary length. Using terminal dilutions of two preparations of genomic DNA, we found a good correlation between the number of capillaries giving positive reactions and the number of capillaries calculated to contain  $\geq 1$  template molecule. The inference of single molecule sensitivity is further supported by the observation of peaks of elevated F/R emission along the length of capillaries estimated to contain 1 or 2 template molecules. Presumably these peaks result from localized accumulation of PCR product.

Is it reasonable to expect that PCR products and degraded TaqMan probe would remain localized on a scale of 5 mm for a few hours after PCR (the time it took us to complete some of the fluorescence assays)? We suspect that the narrowness of the capillaries effectively eliminates convection, so that molecular movement is dominated by diffusion. Molecules the size of completely degraded probe (e.g. rhodamine-dGTP) have diffusion constants of  $\sim 3\text{--}5 \times 10^{-6} \text{ cm}^2/\text{s}$  in water at room temperature (14). The diffusion constant increases with temperature as  $D \sim kT/\eta$ , where  $T$  is measured in Kelvin and  $\eta$ , the viscosity, decreases with temperature (15). The viscosity of water decreases  $\sim 3$ -fold as temperature increases from 25 (298 K) to 92°C (365 K) (16); thus  $D$  would increase  $\sim 3.25$ -fold over this temperature range. The root mean square distance traveled by a molecule with diffusion constant  $D$  in time  $t$  is  $(2Dt)^{1/2}$ , or  $\sim 2\text{--}5$  mm in 2 h for molecules the size of completely degraded probe at temperatures between 25 and 92°C. The PCR product, based on its molecular weight, should have a diffusion constant of  $\sim 0.45 \times 10^{-6} \text{ cm}^2/\text{s}$  and should diffuse about three times less far than degraded probe in the same time. These 'back of the envelope' calculations indicate that the

widths of fluorescent peaks that we observed are consistent with diffusion-mediated spreading of PCR product and degraded probe.

The fluorescence peak widths might be slightly larger than predicted by simple diffusion, due to the tendency of PCR to saturate in regions where the concentration of PCR product is high. So long as all of the amplified molecules were replicated each cycle, the progeny from a single starting template would have the same average displacement as a collection of independent molecules, i.e. they would appear to diffuse with a root mean square displacement that is proportional to the square root of the time (see Appendix). However, as PCR begins to saturate, molecules near the center of the distribution, where concentration is high, have a lower probability of being replicated than molecules near the 'edge' of the distribution, where concentration is low. This unequal probability of replication would tend to make the distribution broader.

The diffusion model suggests that detection of single target molecules by TaqMan assay would be difficult using conventional size capillaries. A 5 mm segment (characteristic diffusion distance for degraded probe) in a 0.8 mm inner diameter capillary contains ~2.5  $\mu$ l. After spreading in one fiftieth of this volume (~50 nl), the fluorescent signal we obtained from single starting molecules in 50  $\mu$ m inner diameter capillaries was sometimes not above the background (see Table 2; average F/R at 24–48 h and number of capillaries in which maximum F/R > 1). Thus, PCR would have to be significantly more efficient than we achieved to detect single molecules in microliter volumes.

While limited diffusion of product and degraded probe was important for our ability to detect single starting molecules in capillaries, diffusion of reactants present in the original reaction mixture is usually not limiting for PCR; for example, at the (conventional) concentration of Taq polymerase used here, the average distance between polymerase molecules is ~1  $\mu$ m and polymerase molecules (mol. wt ~100 kDa) diffuse this distance in ~0.01 s. Thus, all portions of the reaction should be sampled by a polymerase molecule many times each second.

While we were able to confine PCRs to volumes of 3–60 nl by using capillaries with small diameters and relying on the fortuitously slow rate of diffusion, more practical application of these results will come from the development of a device for confining PCRs to small regions in three dimensions. Such devices could in theory be used to measure the number of template molecules in a sample simply by counting the number of positive reactions in replicate PCRs containing terminal dilutions of sample. With the potential for automation and a closed system to prevent carry-over contamination, such a device would have significant promise for clinical applications of PCR. An assay based on presence versus absence of PCR product in replicate reactions may be more robust with respect to small changes in amplification efficiency than quantitative competitive assays or time-to-reach-threshold level assays that require assumptions about relative or absolute replication rates. Several groups have reported other approaches to miniaturization of PCR chambers and instrumentation, including PCR chips (17), devices for combined amplification, electrophoresis and detection (10) and chip-like devices capable of mixing reagents, thermocycling and detecting PCR products (18). To our knowledge, however, this is the first report of successful PCR on the nanoliter scale.

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## APPENDIX

Following the derivation of the diffusion equation in one dimension given in Berg (15), pp. 9–10, suppose each of the  $n$  PCR product molecules present at time  $t$  duplicates and the daughter molecules move a small distance  $\delta$ , either to the left or right, in the short time  $\tau$  after duplication. Letting  $x_j$  designate the displacement of the  $j$ th molecule before duplication, the mean square displacement of the  $2n$  molecules at time  $t + \tau$  is  $\langle x^2 \rangle_{t+\tau} = (1/2n) \sum_{j=1}^n [(x_j \pm \delta)^2 +$

$$(x_j \pm \delta)^2] = (1/n) \sum_{j=1}^n [x_j^2 \pm 2x_j\delta + \delta^2] = \langle x^2 \rangle_t + \delta^2, \text{ where the sum}$$

over the  $\pm 2x_j\delta$  terms averages to zero, since  $+$  and  $-$  are equally likely. Thus, the mean square displacement increases by  $\delta^2$  in time  $\tau$ , which is exactly the result for  $n$  independent particles. Thus, the mean square displacement of the ensemble of progeny molecules derived from a single template is the same as that of the single template or a large number of identical molecules starting at the position of the template. This analysis neglects the fact that formation of a DNA–polymerase complex during replication may transiently decrease the rate of diffusion.